Diagnostic accuracy of haptoglobin within ovarian cyst fluid as a potential point-of-care test for epithelial ovarian cancer: an observational study

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The diagnostic accuracy of haptoglobin within ovarian cyst fluid as a potential point-of-care test for epithelial ovarian cancer: an observational study

Aniza P Mahyuddin*, Lin Liu*, Chanqing Zhao, Kothandaraman Narasimhan, Manuel Salto-Tellez, Brendon Nghee Kheem Pang, Diana Gkeok Stzuan Lim, Loganath Annalamai, Jerry K Y Chan, Timothy Yong Kuei Lim, Arijit Biswas, Gregory Rice, Khalil Razvi#, Mahesh Choolani#.

Aniza P Mahyuddin
Department of Obstetrics & Gynaecology,
National University of Singapore,
NUHS Tower Block, Level 12,
1E Kent Ridge Road,
Singapore 119228.
E-mail: obgapm@nus.edu.sg

Lin Liu
Murdoch Childrens Research Institute,
3rd Floor South Building,
Royal Children's Hospital
Flemington Road, Parkville, Victoria 3052 Australia.
E-mail: lin.liu@mcri.edu.au

Changqing Zhao
Boehringer Ingelheim (S) Pte Ltd,
37-00, The Concourse,
Singapore.
E-mail: zhaoosme@gmail.com

Narasimhan Kothandaraman
Department of Obstetrics & Gynaecology,
National University of Singapore,
NUHS Tower Block, Level 12,
1E Kent Ridge Road,

Singapore 119228.
E-mail: obgkn@yahoo.com

Manuel Salto-Tellez
Centre for Cancer Research and Cell Biology,

Room 01.003, CCRCB Block 5 MBC Site,

Queen's University, 97 Lisburn Road,

Belfast BT9 7AE, Northern Ireland,

United Kingdom.

Email: m.salto-tellez@qub.ac.uk

Brendon Nghee Kheem Pang

Department of Pathology,
National University Hospital,
5, Lower Kent Ridge Road,
1 Main Building, Level 3,
Singapore 119074.

Email: brendan_nk_pang@nuhs.edu.sg

Diana Gkeok Stzuan Lim

Department of Pathology,
National University Hospital,
5, Lower Kent Ridge Road,
1 Main Building, Level 3,
Singapore 119074.

Email: diana_gz_lim@nuhs.edu.sg

Loganath Annalamai

Department of Obstetrics & Gynaecology,
National University of Singapore,
NUHS Tower Block, Level 12,
1E Kent Ridge Road,
Singapore 119228.
E-mail: Retired. No contact details.

Jerry KY Chan
Department of Reproductive Medicine,
KK Women's and Children's Hospital,
100 Bukit Timah Road,
Singapore 229899.
E-mail: jerrychan@duke-nus.edu.sg

Timothy Yong Kuei Lim
Department of Gynaecological Oncology,

KK Women's and Children's Hospital,

100 Bukit Timah Road,

Singapore 229899.

E-mail: timothy.lim.yk@kkh.com.sg

Arijit Biswas
Department of Obstetrics & Gynaecology,
National University of Singapore,
NUHS Tower Block, Level 12,
1E Kent Ridge Road,
Singapore 119228.
E-mail: arijit_biswas@nuhs.edu.sg

Gregory Rice
University of Queensland Centre for Clinical Research,
Building 71/918,
Royal Brisbane & Women's Hospital Campus,
The University of Queensland, Herston, QLD, 4029 Australia.
E-mail: g.rice@uq.edu.au

Khalil Razvi
Department of Obstetrics & Gynaecology,
Southend University Hospital NHS Foundation Trust,
Westcliff-on-Sea, England, SS0 0RY.
E-mail: khalil.razvi@southend.nhs.uk

Mahesh Choolani,
Department of Obstetrics & Gynaecology,
National University of Singapore,
NUHS Tower Block, Level 12,
1E Kent Ridge Road,
Singapore 119228.
E-mail: obgmac@nus.edu.sg

Correspondence:
Mahesh Choolani,
Department of Obstetrics & Gynaecology,
National University of Singapore,

NUHS Tower Block, Level 12,
1E Kent Ridge Road,
Singapore 119228.
E-mail: obgmac@nus.edu.sg
Tel: +65 6772 2672 Fax: +65 6779 4753

*Aniza P Mahyuddin and Lin Liu are joint first authors and contributed equally to this study.

Khalil Razvi and Mahesh Choolani are joint last authors.

Running title: Haptoglobin: point-of-care diagnostics for ovarian cancer

ABSTRACT

Objective: To investigate haptoglobin within ovarian cyst fluid (OCF) as a diagnostic biomarker for epithelial ovarian cancer (EOC) and develop an in vitro diagnostic point-of-care device test (IVDPCT) for use in the operating theatre.

Design: Retrospective and prospective cohort study

Setting: South-East-Asia

Population: Women with suspicious ovarian cysts

Methods: Proteomic, immunohistochemical and ELISA methods measured haptoglobin in OCF to differentiate benign and EOCs. Diagnostic performance of haptoglobin was compared to CA125, Risk Malignancy Indices (RMI) and frozen section. Blinded validation of the IVDPCT was performed.

Main outcome measures: Prediction of malignancy

Results: In patients with benign cysts (n=87) haptoglobin concentration measured by ELISA was 0·70±0·09 mg/ml; early stage-EOC (n=17) was 6·22±0·53 mg/ml; and late stage-EOC (n=20) was 6·57±0·65 mg/ml. Haptoglobin in EOCs was significantly higher than benign

cysts (P<0·0001). Haptoglobin using rapid colorimetric assay (RCA) on a training set had sensitivity of 97·3% and specificity 92·0%, comparable to ELISA and frozen sections. The haptoglobin AUROC curve was 0·999 (95%CI 0·997-1·000) compared to 0·895 (95%CI 0·814-0·977, P<0·05) for CA125. Haptoglobin performed significantly better than all the RMIs (P<0·01). Blinded validation studies showed a minor drop in average diagnostic performance (sensitivity 85·2% and specificity 90·5%) compared to training set. However, when compared to frozen section, haptoglobin was no worse in diagnostic accuracy for malignancy.

Conclusion: Haptoglobin was identified as a biomarker for the detection of EOC with potential as a point-of-care diagnostic tool.

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Keywords: epithelial ovarian cancer, in vitro diagnostic, biomarker, haptoglobin, point-of-care, frozen section

Tweetable abstract: Haptoglobin within ovarian cyst fluid: a biomarker for epithelial ovarian cancer and point-of-care diagnostics

INTRODUCTION

Epithelial ovarian cancer (EOC) is a disease with poor prognosis. Lifetime risk of developing EOC is 1·4%, and worldwide 239,000 new cases are diagnosed annually.[1] Two key factors that could improve survival in EOC are early diagnosis and appropriate primary surgery.[2] Most patients are diagnosed at late stage (LS-EOC), when the tumours respond poorly to therapy and have metastasised.[3] Consequently, five-year survival rates are only 40%.[4] In contrast, patients diagnosed with early-stage EOC (ES-EOC) have 5-year survival rates >90%.[5] There have been attempts to define symptoms that warrant earlier investigation, however, EOC symptom-complexes have low positive-predictive values (PPV) for ES-EOC[6], and one in six ES-EOC patients are asymptomatic.[7] No clinically-proven effective screening exists.[8] There may be a role for a multimodal screening strategy[4,9] however, the value of screening in lowering mortality may only be realised after protracted close surveillance.[4]

Benign ovarian cysts are common[10] and the relative preponderance of cancers versus benign cysts vary.[4,11,12] The natural history of cysts remains poorly understood.[9,13] Laparoscopy or laparotomy is the standard surgical approach for benign ovarian masses,[8] but in up to 14%, an unexpected malignancy is encountered.[13] Preoperative prediction of the malignant potential remains challenging in early stage disease. This is especially true for ovarian masses identified in premenopausal women, and in South-East Asian countries where the incidence of EOC rises in the thirties.[8,14-16] Intraoperative frozen section (FS) has been proposed to prevent delay in primary surgery,[17] but suffers from several

limitations such as wide variation in accuracy due to large size of cysts, sampling error, and limitation of available staining methods owing to time constraints[18,19]

Our pre-clinical exploratory proteomic studies demonstrated the presence of haptoglobin within the OCF which was able to differentiate between benign ovarian tumours, borderline and EOC (detailed in results and Appendix S1). As an acute phase reactant protein, serum haptoglobin concentrations rise in many cancers, but with poor sensitivities and specificities. In contrast, we showed through tissue microarray studies that haptoglobin within OCF accurately reflects the benign and malignant nature of the ovarian cystic masses since it is produced and concentrated locally within the cancer (detailed in results).

We hypothesised that haptoglobin may be of potential use as an in vitro diagnostic tool with the development and validation of a clinical assay using retrospective and prospective clinical samples.

MATERIALS AND METHODS

Subject recruitment and sample collection

We recruited 324 patients (Fig. 1A), between the ages of 11-74 years between December 2003 and March 2014 undergoing either laparoscopy or laparotomy for clinically suspicious tumours of the ovary at our local academic medical centre (LMC) and from five hospitals in Indonesia and Vietnam (regional medical centres, RMCs).

Our local academic medical centre recruited 284 patients (172 benign, 30 borderline and 82 malignant tumours), a combination of retrospective and prospective cohorts. In our retrospective cohort, we collected and archived ovarian cyst fluid from 184 patients. The

histopathology diagnoses (frozen section, when conducted, and paraffin) for the 184 samples were known during selection for the type(s) of analysis that were performed. Retrospective sampling was performed to facilitate batch testing in laboratory investigations (preclinical and assay development phase). Prospective consecutive sampling was performed in 100 patients and analysed without any prior knowledge of the histopathology diagnosis (prospective validation phase). The RMC recruited 40 patients, and these samples were combined with our local retrospective cohort (31 benign tumours and 9 malignant tumours). In aggregate, they represented 17·9% of the whole retrospective cohort used.

The National Healthcare Group Domain Specific Review Board approved local collection and use of patient samples, and the local ethics committee at each regional centre gave corresponding approval. All patients gave written informed consent. All tumours were defined by histopathological diagnoses (reference standard) and staging was according to the International Federation of Gynecology and Obstetrics (FIGO) staging for ovarian cancer.[20] Ovarian dermoid cysts, endometriotic cysts, pelvic inflammatory disease, tubo-ovarian abscesses, solid and frank malignant tumours were excluded from this study.

Ovarian cyst fluid samples were collected either at laparoscopy or laparotomy without spillage into the abdominal cavity. At laparotomy, the intact cyst was removed from the abdominal cavity followed by immediate aspiration of cyst contents via a syringe. At laparoscopy, the cyst was contained within an endobag and aspiration of the cyst was performed either from the endobag before or after being removed from the abdominal cavity. OCF that were heavily contaminated with blood were excluded as they contain haptoglobin and haemoglobin, which would interfere with our haptoglobin-based test. Retrospective samples were transported on ice to the laboratory, centrifuged at 2,000 *x g* for 10 min at 4°C and the supernatant stored in -80°C until analysis. Prospective samples are kept on ice and analysed immediately, and not exceeding 4 hours post-cyst aspiration.

Measurement of haptoglobin concentrations by ELISA and rapid colorimetric assay (RCA) on retrospective samples

The OCF haptoglobin concentrations were measured in benign tumours, and in ES- and LS-EOCs using ELISA (Appendix S1) on 144 retrospective OCF samples (Fig. 1A). Histopathological classifications of all tumours (reference standard) and FIGO stages of malignant cases are detailed in Table S1 and S2. We used an RCA, and semi-quantitatively measured haptoglobin concentrations on the same retrospective cohort (Appendix S1). A receiver operating characteristic (ROC) curve was used to obtain haptoglobin cut-off concentrations (data not shown).

Comparing OCF haptoglobin with CA125 and Risk of Malignancy Indices (RMIs) for prediction of malignancy

Preoperative ultrasound reports and CA125 levels of patients recruited from LMC (Table S3) were obtained and evaluated for the risk of ovarian cancer by calculating three RMIs (RMI 1[21], RMI 2[22] and, RMI 3[23]; Appendix S1).

Validation of OCF haptoglobin in blinded retrospective and prospective real-time samples

We performed validation studies on 150 OCF samples (Fig. 1A, n=50 retrospective and n=100 prospective) in 96-well plate format (index test) (RCA) and IVDPCT format (index test) (RCA in IVDPCT; Development of IVDPCT- Appendix S1, Fig. S3) both in the laboratory and in the operating theatre (Appendix S1). The reference/gold standard for diagnosis of all tumours was tissue histopathology. Independent testers performed the index tests in the laboratory and operating theatre. The IVDPCT testers received training on IVDPCT use. Testers watched a training video of IVDPCT use followed by a live

demostration of the IVDPCT. Next, the testers performed practicals using the IVPCT with known haptoglobin controls.

Statistical analysis

We tested data for normality with D'Agostino's K^2 test, and evaluated statistical significance with Student *t* test (two-tailed) for comparison of age and peak intensity; Mann-Whitney *U* test (two-tailed) for comparison of serum CA125 level, tissue expression of haptoglobin, and OCF haptoglobin concentration; and Fisher's exact test (two-tailed) or Pearson's chi-square test for comparison of the proportions of patients present with each ultrasound feature or score between benign and malignant EOCs. We used ROC curves to assess the performance of biomarkers and evaluated the statistical significance for the comparison of two areas under the curve.[24] We used nonparametric Spearman and Bland-Altman for RCA and IVDPCT correlation tests. All statistical analyses were performed using GraphPad Prism 5.03 (GraphPad Software, La Jolla, CA, USA) and MedCalc v11.2.1.0 (MedCalc Software bvba, Mariakerke, Belgium). We expressed data as mean±S.E.M. and considered results statistically significant at $P<0.05$.

RESULTS

The results are presented in three phases: Preclinical Exploratory, Clinical Assay Development, and Prospective Validation studies.

Preclinical Exploratory phase: we present results of haptoglobin, a potential biomarker within OCF, identified through proteomics (mass spectrometry, SDS-PAGE, SELDI western blot) and immunocytochemical (tissue microarray) methods.

We analysed retrospective OCF (Fig. 1A) from benign ovarian tumours, ES-EOCs and LS-EOCs with SELDI-TOF/MS in mass ranges 7-60 kDa and identified a high-intensity discriminatory peak at 17.5 kDa that varied in proportion according to the extent of malignancy. It was present in 9/10 LS-EOCs, but absent/present at very low intensities in benign tumours. In early-stage disease, this peak was intermediate between benign and late-stage disease (Fig. S1A). Haptoglobin identity was confirmed by SDS-PAGE, peptide sequence matching, SELDI and western blot (Appendix S1; Fig. S1B-F; S2A,B).

Using immunohistochemistry on tissue microarray (TMA) slides, we observed strong expression within the epithelial lining cells of EOC and absent/weak expression of haptoglobin in the epithelial linings of normal ovaries and benign ovarian cysts (Fig. S2C). These images were converted to grey-scale luminosity grading. Here we showed that cancerous cells had significantly higher levels of haptoglobin expression than seen in benign tumour cells ($P<0.0001$) (Fig. S2D), and borderline cells have intermediate staining.

Clinical Assay Development phase: we present ELISA (quantitative) and rapid colorimetric assay (semi-quantitative) measurements of haptoglobin between benign, borderline, ES- and LS-EOCs. Result of operating characteristics of haptoglobin versus CA125 and RMI's is shown. Results of assay optimisation, observer bias study and reproducibility in 96-well plate and IVDPCT are presented.

Detection of OCF haptoglobin concentration by ELISA and RCA.

The OCF haptoglobin concentration detected by ELISA in women with ES- or LS-EOC (6·22±0·53 mg/ml and 6·57±0·65 mg/ml, respectively, $P>0·05$) was significantly higher compared to that in women with benign tumours (0·70±0·09 mg/ml)($P<0·0001$), although there was no significant difference in haptoglobin concentrations between ES-EOCs and LS-EOCs (Fig. 1B). Amongst the women diagnosed with ES-EOC, five had serous carcinoma subtype (three low/intermediate-grade and two high-grade serous carcinoma (HGSC)). In all these samples, haptoglobin concentrations (range 3.9-7.3 mg/ml) were higher than the cut-off value (2.85 mg/ml). The accuracy of RCA (at cut-off 0.5 mg/ml) was similar to that of ELISA (at cut-off 2.85 mg/ml) (Table 1) as determined using retrospective OCF samples (n=124; Fig. 1A, Table S1). Using an ELISA-based assay, haptoglobin has an overall sensitivity of 97·3% and NPV of 98·8% (Table 1). When considering only retrospective regional medical centre samples, in resource-challenged countries, the sensitivity and NPV are 88·9% and 96·9%, respectively. Using an RCA where the results could be ready in five minutes inside the operating theatre, the overall sensitivity and NPV were 97·3% and 98·8%, respectively (Table 1). Figure S4 demonstrates the appearance of the RCA in a 96-well plate.

Of the 84 retrospective local academic medical centre cases, 33 women had FSs performed, with 16 benign and 17 cancerous cases as confirmed by histopathology. The overall accuracy in determination of the status of malignancy was 97·0% for cyst fluid haptoglobin determined by ELISA, 93·9% by RCA, and 90·9% for FS (Table 2A). Three cases (9·1%) had FS diagnosis discordant with the histopathology. There was one ES-EOC that FS misdiagnosed as benign (false negative), and two benign tumours misdiagnosed as borderline tumours (false positives). In contrast, in one case (3·0%) OCF haptoglobin measurements by ELISA and in two cases by RCA (6·1%) were discordant with histopathology. All discordant cases were benign tumours being over-diagnosed. Overall,

sensitivity, specificity, PPV and NPV of both methods of detecting OCF haptoglobin were similar to FS (Table 2A).

OCF haptoglobin is more accurate than CA125 or RMIs.

RMI is used frequently in the preoperative triage of patients with ovarian cysts. Menopause status is one key discriminant used in this risk calculation. In the Caucasian population, >80% of EOC patients are postmenopausal. In our local academic medical centre cohort, the mean ages for the benign and malignant groups were 39·6±13·9 and 51·5±10·0 respectively (P<0·0001), but 50% (14/28) of patients with cancer were premenopausal, which lead to lower RMI scores (Table S3). Another key component of the RMI is the CA125 concentration. In our cohort, ten percent of women with benign cysts had elevated CA125 concentrations and 14·3% of patients with malignant disease had a CA125<35 U/ml. Using a ROC curve analysis (Fig. 1C), we show that haptoglobin had superior diagnostic efficiency (AUC 0·999, 95%CI 0·997-1·000) compared with CA125 (AUC 0·895, 95%CI 0·814-0·977; percentageΔ=10·4%; P=0·013). Three versions of the RMI have been used, which are similar in principle (menopausal status, ultrasound features and CA125 concentrations), but differ in the weights of each element: RMI 1[21], RMI 2[22] and, RMI 3[23]. Here too, haptoglobin out-performed all three RMIs (Fig. 1C).

Assay optimisation, observer bias study and reproducibility in 96-well plate and IVDPCT

In the laboratory, we tested RCA in 96-well plates (Fig. S4) and IVDPCT formats (Fig. S3) using 50 retrospective samples (Fig. 1A, Table S5) where histopathology diagnoses were blinded to the testers (Appendix S1). The IVDPCT was in agreement with that of 96-well plate, as indicated by absorbance (630 nm) (Spearman's slope=1·25, R^2=0·961; Bland-Altman (BA) bias= -0·08 (Fig. S5A,B)). There was no significant difference between the mean absorbance for the 96-well and for the IVDPCT (P>0·05; Table S4). There was no interobserver difference in the discrimination of visual colour appearance between the 96-well and IVDPCT (BA bias=0; Fig. S5*C*) and good agreement of visual calls compared to histopathology diagnoses (BA bias=0·01; Fig. S5D) with sensitivity of 100%, specificity 96·8%, PPV 95%, and NPV 100% (Table 3).

Prospective Validation phase: we present sensitivity, specificity, PPV and NPV results of 100 prospective blinded OCF sample assayed in the laboratory and operating theatre.

Fifty consecutive prospective blinded samples were collected at surgery for ovarian cyst and tested in the laboratory using the RCA (index test) (Fig. 1A) and compared to histopathology diagnosis (reference standard; Table S5). We observed sensitivity of 85·7% (95%CI 62·6-96·2%), specificity of 89·7% (95%CI 71·5-97·3%), PPV of 85·7% (95%CI 62·6-96·2%), and NPV of 89·7% (95%CI 71·5-97·3%)(Table 3).

We followed this up with a prospective blinded study of the IVDPCT (index test) with 50 OCF consecutively sampled and tested real-time (Fig. 1A) in the operation theatre with the IVDPCT tester blinded to the type of surgery performed and diagnosis. We recorded visual calls from the IVDPCT and compared to FS and

histopathology (reference standard obtained 1-2 weeks post-surgery; Table S5). The IVDPCT had sensitivity of 70% (95%CI: 35·4-91·9%), specificity of 85% (95%CI: 69·5-93·8%), PPV of 53·8% (95%CI: 26·1-79·6%), and NPV of 91·9% (95%CI: 77-97·9%)(Table 3). Of the 50 IVDPCT cases tested in theatre, 26 had FSs performed, with 22 benign and 4 cancerous cases as confirmed by histopathology. The overall accuracy in determination of the status of malignancy was 92·3% for IVDPCT compared to 88% for FS (Table 2B). Three cases (11·5%) had FS diagnosis incompatible with histopathology and one FS without a diagnosis due to technical difficulties during tissue cryosectioning (IVDPCT result matched histopathology). Three benign tumours were misdiagnosed: one low-grade neoplasm and two borderline tumours (false positives). In contrast, two cases (7·7%) by IVDPCT had OCF haptoglobin measurements discordant with histopathology. All discordant cases were benign tumours being over-diagnosed (false positives). Overall diagnostic performance was higher in IVDPCT when compared to FS (Table 2B).

DISCUSSION

Main findings

We demonstrated the presence of haptoglobin in OCF of benign, borderline and malignant EOCs. The concentration of haptoglobin was significantly raised in ES and LS-EOC compared to benign tumours. We also observed raised haptoglobin concentrations in OCF of low-volume HGSC's.

Semi-quantitative measurements of haptoglobin using a RCA in clinical assay development gave diagnostic accuracies similar to ELISA and frozen section. The haptoglobin AUROC curve was 0·999 (95%CI 0·997-1·000) compared to 0·895

(95%CI 0·814-0·977, P<0·05) for CA125. Haptoglobin performed significantly better than all the RMIs (P<0·01). We showed good correlation and between the RCA and IVDPCT and minimal intra- and inter-observer bias.

In our prospective validation study, the diagnostic accuracy of IVDPCT was marginally poorer compared to that of the assay development tests, but it does maintain accuracy which is comparable to FS.

Strengths and limitations

Several studies exploring OCF for biomarker analysis found it to have little use for discriminating between benign and malignant tumours.[25,26] One study suggested that OCF contained tumour-specific biomarkers,[25] but this study had several limitations. The putative biomarkers identified by protein profiling were not confirmed by analysis of the exact peptide sequence; none of the peaks were superior to CA125 used alone; and a 1:1 ratio of benign versus malignant samples were used leading to a discovery strategy that would yield low PPVs.[27]

We analysed haptoglobin concentrations in 324 OCF samples and haptoglobin is able to differentiate benign from malignant EOCs with high accuracy. Only haptoglobin within borderline ovarian tumour samples showed poorer diagnostic accuracy for malignancy when compared to FS, and haptoglobin's accuracy was only marginally better compared to CA125 or RMI1 (Table S2). A recent Cochrane review highlighted similar problems with the accuracy of frozen section in the diagnosis of borderline ovarian tumours.[19] This validated our FS findings of poorer

accuracy (80%) in borderline ovarian tumours when compared to paraffin histopathology (Table S2).

Tissue microarray study of 141 ovarian tissue samples showed haptoglobin accumulation within ovarian cyst fluid is more reflective of the biological changes within the tumour microenvironment. Although the pathophysiology of haptoglobin overexpression within malignant cysts remains unclear, we showed specific overexpression within the epithelial lining cells of cancerous cysts (Fig. S2C). While some investigators have shown presence of haptoglobin-1 precursor (HAP1) within ovarian cancer cells,[28] other acute phase reactants have also been reportedly raised in HGSC's.[29]

Unlike the RMIs, haptoglobin-based segregation for malignancy was independent of the patient's menopausal status. Here, haptoglobin performed significantly better when compared with either CA125 used alone, or as part of the RMIs. This is because in the western hemisphere, EOC is a disease mainly in the postmenopausal women.[21] In contrast; half of the women with EOC in our study were premenopausal. This is in keeping with national data from Singapore (National Cancer Registry), and with our own hospital data collected over three years, suggesting an earlier rise in the incidence of EOC locally and regionally.[14-16] The sensitivity of RMI1 for adnexal masses in this local hospital cohort of Asian patients is only 54%. Recent use of the IOTA risk prediction model has shown a higher accuracy than RMI in both pre- and post-menopausal women, but still does not remove the need for intraoperative diagnostics in evaluation of suspicious ovarian tumours.[30]

EOC prevalence in our study ranged between 20% and 42%, which reflects clinical practice, as there are greater numbers of benign compared to malignant cases undergoing investigation. Even with the increased number of benign samples tested, haptoglobin gave high positive predictive values (PPV's) (range 83.7% to 94.7%) in all OCF samples. Typically, estimates of diagnostic performance in the validation samples are more realistic (and lower) than in the clinical assay development samples and this is seen in the lowered diagnostic performance of the validation samples. Due to the smaller sample size and lower prevalence of EOC, the confidence interval for the validation set is wider, compared to the assay development samples.

Interpretation

In our study, haptoglobin and FS seem to have comparable diagnostic accuracies. Sensitivity of FS varies between 65-97%, with specificities of 97-100% according to a meta-analysis.[18] Factors contributing to this wide variation in accuracy include large size of cysts, sampling error, and limitation of available staining methods owing to time constraints.[18,19] The overall utility of FS is even worse because of additional logistical limitations, and it can create inefficiencies in operating room utilisation with surgeries pausing for >30 minutes, increasing patient morbidity. Haptoglobin however, has the additional benefit of being a rapid, easy, and cost-efficient diagnostic test to perform at the patient's point-of-care.

The IVDPCT is intended for use in women undergoing surgical investigation for a suspicious cystic mass, as a triage for FS, or even as a surrogate for FS in resource-challenged countries where FS is not available. In a test with a high NPV, such as

this, if the IVDPCT were negative, we could avoid FS and complete the surgery; a true positive test would require either a FS or involvement of gynaecologic oncologists if FS were not readily available. Initial surgery performed by a gynaecologic oncologist is associated with accurate staging, complete resection of disease and improved patient prognosis.[31,32]

Conclusion and research recommendations

The present study is the first demonstration of OCF haptoglobin as a highly discriminatory biomarker between benign and malignant EOCs. Larger prospective blinded clinical studies are required to determine the operating characteristics of the IVDPCT in a relevant population to determine detection and false negative rates. Larger trials are also needed in identifying malignant cases and selecting benign cases that would not require FS evaluation. Our data suggests the potential utility of OCF haptoglobin as an in vitro diagnostic point-of-care tool with accuracy comparable to frozen section.

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Disclosure of interests

The National University of Singapore (NUS) has licensed the patents out to INEX Innovations Exchange Pte Ltd, a local company spun out from the university by three of the authors - AB, KR and MC. MC, holds shares in, and is a non-executive director of INEX Innovations Exchange Pte Ltd. KR and AB are shareholders of INEX Innovations Exchange Pte Ltd. All other authors declare no conflict of interest. The ICMJE disclosure forms are available as online supporting information.

Contribution to authorship

LL, APM and MC designed experiments, analysed data and wrote the manuscript. GR, KN, LA and CZ assisted with proteomic study. TLYK and JKYC contributed key reagents and advice. MST, DGSL and BNKP performed the tissue microarray study. AB, KR and MC conceived and supervised the project. All authors provided critical comments and editorial changes.

Ethics approval

The National Healthcare Group Domain Specific Review Board, Singapore (D/00/856 - 8th June 2000); DSRB2007/00240 - July 2007) approved local collection and use of patient samples, and the local ethics committee at each regional centre gave corresponding approval. All patients gave written informed consent.

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TABLES

Table 1 Clinical assay development: sensitivity, specificity, PPV and NPV of cyst fluid haptoglobin determined by ELISA and rapid colorimetric assay in the differential diagnosis of benign and malignant epithelial ovarian tumours in retrospective archived samples from 124 women.

	Local academic medical centre, N=84					
	Haptoglobin by ELISA*			Haptoglobin by rapid colorimetric assay**		
	CA	Non-CA	Total	CA	Non-CA	Total
Test +	28	2	30	27	3	30
Test -	0	54	54	1	53	54
Total	28	56	84	28	56	84
	Value (%)	95% Confidence Interval		Value (%)	95% Confidence Interval	
		Lower	Upper		Lower	Upper
Sensitivity	100	85·0	100	96·4	79·8	99·8
Specificity	96·4	86·6	99·4	94·6	84·2	98·6
PPV	93·3	76·5	98·8	90·0	72·3	97·4
NPV	100	91·7	100	98·1	88·8	99·9
	Regional, N=40					
	Haptoglobin by ELISA*			Haptoglobin by rapid colorimetric assay**		
	CA	Non-CA	Total	CA	Non-CA	Total
Test +	8	0	8	9	4	13
Test -	1	31	32	0	27	27
Total	9	31	40	9	31	40
	Value (%)	95% Confidence Interval		Value (%)	95% Confidence Interval	
		Lower	Upper		Lower	Upper
Sensitivity	88·9	50·7	99·4	100	62·9	100
Specificity	100	86·3	100	87·1	69·2	95·8
PPV	100	59·8	100	69·2	38·9	89·6
NPV	96·9	82·0	99·8	100	84·5	100
	Combined local and regional, N=124					
	Haptoglobin by ELISA*			Haptoglobin by rapid colorimetric assay**		
	CA	Non-CA	Total	CA	Non-CA	Total
Test +	36	2	38	36	7	43
Test -	1	85	86	1	80	81
Total	37	87	124	37	87	124
	Value (%)	95% Confidence Interval		Value (%)	95% Confidence Interval	
		Lower	Upper		Lower	Upper
Sensitivity	97·3	84·2	99·9	97·3	84·2	99·9
Specificity	97·7	91·1	99·6	92·0	83·6	96·4
PPV	94·7	80·9	99·1	83·7	68·7	92·7
NPV	98·8	92·8	99·9	98·8	92·4	99·9

*Haptoglobin at cut-off 2·85 mg/ml; ** Haptoglobin at cut-off 0·5 mg/ml. CA-Cancer; Non-CA Non-Cancer; PPV - positive predictive value; NPV - negative predictive value

Table 2 Diagnostic accuracy of index test versus frozen section in clinic assay development and prospective validation phase. (A) Clinical assay development phase: ELISA, rapid colorimetric assay and frozen section compared to histology for the diagnosis of epithelial ovarian tumours in a subset of women (n=33) (B) Prospective validation (in operating theatre): IVDPCT and frozen section compared to histology for the diagnosis of epithelial ovarian tumours in a subset of women (n=26).

A.	Haptoglobin by ELISA*			Haptoglobin by rapid colorimetric assay**			Frozen section		
	CA	Non-CA	Total	CA	Non-CA	Total	CA	Non-CA	Total
Test +	17	1	18	17	2	19	16	2	18
Test -	0	15	15	0	14	14	1	14	15
Total	17	16	33	17	16	33	17	16	33
	Value (%)	95% Confidence Interval (%)		Value (%)	95% Confidence Interval (%)		Value (%)	95% Confidence Interval (%)	
		Lower	Upper		Lower	Upper		Lower	Upper
Sensitivity	100	77·1	100	100	77·1	100	94·1	69·2	99·7
Specificity	93·8	67·7	99·7	87·5	60·4	97·8	87·5	60·4	97·8
PPV	94·4	70·6	99·7	89·5	65·5	98·2	88·9	63·9	98·1
NPV	100	74·7	100	100	73·2	100	93·3	66·0	99·7
Accuracy	97			93·9			90·9		

B.	IVDPCT in theatre			Frozen section		
	CA	Non-CA	Total	CA	Non-CA	Total
Test +	4	2	6	4	3	7
Test -	0	20	20	0	18	18
Total	4	22	26	4	21	25**
	Value (%)	95% Confidence Interval (%)		Value (%)	95% Confidence Interval (%)	
		Lower	Upper		Lower	Upper
Sensitivity	100·0	39·6	100·0	100·0	39.6	100·0
Specificity	90·9	69·4	98·4	85·7	62·6	96·2
PPV	66·7	24·1	94·0	57·1	20·2	88·2
NPV	100·0	80·0	100·0	100·0	78·1	100·0
Accuracy	92·3			88·0		

*Haptoglobin at cut-off 2·85 mg/ml; ** Haptoglobin at cut-off 0·5 mg/ml; PPV - positive predictive value; NPV - negative predictive value; IVDPCT - in vitro diagnostic point-of-care test device.

**One FS did not provide a diagnosis due to technical difficulties during cryosectioning whereas IVDPCT and histology results were concordant.

Table 3 Sensitivity, specificity, PPV and NPV of cyst fluid haptoglobin determined by RCA and IVDPCT in the differential diagnosis of benign and malignant epithelial ovarian tumours in 150 blinded samples (clinical assay development phase (n=50) and prospective validation phase (n=100)).

	RCA (n=50)			**IVDPCT in lab** (n=50)			**IVDPCT in theatre** (n=50)		
	CA	**Non-CA**	**Total**	**CA**	**Non-CA**	**Total**	**CA**	**Non-CA**	**Total**
Test +	18	3	21	19	1	20	7	6	13
Test -	3	26	29	0	30	30	3	34	37
Total	21	29	50	19	31	50	10	40	50
	Value (%)	**95% Confidence Interval (%)**		**Value (%)**	**95% Confidence Interval (%)**		**Value (%)**	**95% Confidence Interval (%)**	
		Lower	**Upper**		**Lower**	**Upper**		**Lower**	**Upper**
Prevalence	42·0%			38·0%			20·0%		
Sensitivity	85·7%	62·6%	96·2%	100·0%	79·1%	100·0%	70·0%	35·4%	91·9%
Specificity	89·7%	71·5%	97·3%	96·8%	81·5%	99·8%	85·0%	69·5%	93·8%
PPV	85·7%	62·6%	96·2%	95·0%	73·1%	99·7%	53·8%	26·1%	79·6%
NPV	89·7%	71·5%	97·3%	100·0%	85·9%	100·0%	91·9%	77·0%	97·9%
Accuracy	88·0%			98·0%			82·0%		

Haptoglobin at cut-off 0·5 mg/ml; RCA-rapid colorimetric assay; IVDPCT-in vitro diagnostic point-of-care test device; CA-Cancer; Non-CA Non-Cancer; PPV-positive predictive value; NPV-negative predictive value

Figure 1.

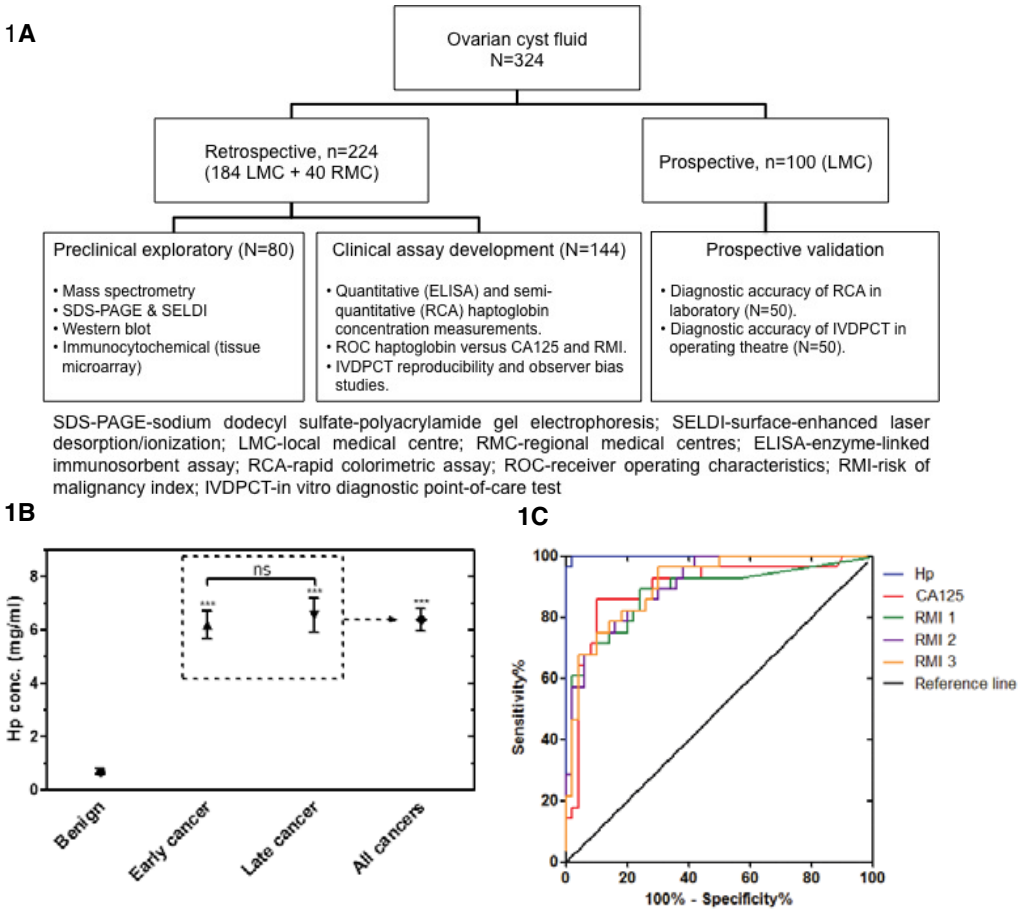

Figure 1 Haptoglobin levels are increased in ovarian cancer cyst fluid (CF). (A) Distribution and use of retrospective and prospective OCF samples for preclinical exploratory, clinical assay development and prospective validation study. (B) ELISA for ovarian cyst fluid (OCF) haptoglobin in 87 benign ovarian tumours, 17 early and 20 late stage ovarian cancers. Data are mean± S.E.M. *** P< 0·0001 versus benign. ns=non-significance (P = 0·98). (C) ROC curve analysis showing the relationship between sensitivity and specificity for CF haptoglobin (blue line), serum CA125 (red line), RMI 1 (green line), RMI 2 (purple line) and RMI 3 (orange line) in the discrimination between benign tumours (n = 50; six benign cases without serum CA125 information were excluded from ROC analysis) and ovarian cancers (n = 28). Haptoglobin performed better than CA125 (P = 0·013), RMI 1 (AUC 0·879, 95% CI 0·793-0·966; P = 0·007), RMI 2 (AUC 0·906; 95% CI 0·843-0·970; P = 0·005) and RMI 3 (AUC 0·909, 95% CI 0·846-0·973; P = 0·006). At a haptoglobin cut-off level of 2·85 mg/ml the sensitivity was 97·3% (95% CI 84·2-99·9%), specificity was 97·7% (95% CI 91·1-99·6%), PPV was 94·7% (95% CI 80·9-99·1%) and NPV was 98·8% (95% CI 92·8-99·9%), at a prevalence of 3:2 benign to malignant cases.